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"A Better Way to Excise Inhibitory Molecules (CSPGs) from a Spinal Cord Injury Scar to Promote Regeneration"

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14. ABSTRACT A major consequence of spinal cord injury (SCI) is the development of a glial scar. Although the scar has benefits for tissue repair, it also blocks neural regeneration. Inhibitory chondroitin sulfate proteoglycans (CSPGs) are elevated in the glial scar and are a major deterrent to successful regeneration. In the present study, we have demonstrated that injured astrocytes produce a wide variety of inhibitory CSPGs. To develop a more efficient method to accomplish CSPG degradation (than the bacterial enzyme chondroitinase), we are addressing a normally occurring catabolic protein for CSPG degradation, the neural aggrecanase, ADAMTS-4. We employed a two-prong approach, using studies both <i>in vitro</i> , and <i>in vivo</i> . We produced recombinant ADAMTS-4 protein and used this enzyme to reduce CSPG inhibition in experiments both <i>in-vitro</i> , and <i>in vivo</i> . We have developed critical reagents (lentivirus) and methods, and have tested these in proof of principle assays, as well as in a dorsal SCI in a rodent model. We are conducting behavioral assessments to validate the success of aggrecanase treatment, and have data indicating that aggrecanase successful ameliorates some of the negative consequences of dorsal SCI injury. The final stage of this study (no cost extension) will be to repeat the aggrecanase tests, combining it with chondroitinase ABC. The significance of these studies is the development of an efficient means by which to attenuate axonal inhibition, and thereby promote plasticity and regeneration of adult neurons following SCI.					
15. SUBJECT TERMS astrocytic scar; CSPGs; recombinant ADAMTS-4; dorsal hemisection model; pellet retrieval task					
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Introduction

Subject. Spinal cord injury (SCI) is a devastating condition affecting as many as 306,000 individuals in the US alone (<http://www.brainandspinalcord.org/spinal-cord-injury/statistics.html>; as of Oct. 28, 2013). Beyond US soil, SCI is an all-too-common result of military combat – all with an enormous emotional, social, and financial cost to individuals and to society. Despite much needed attention over the past few decades and some significant advances, the cellular and molecular mechanisms leading to SCI are not yet clear. **Purpose:** A major consequence of SCI is the development of an astrocytic glial scar. Although the scar has benefits for tissue repair, it also blocks neural regeneration. Inhibitory **chondroitin sulfate proteoglycans (CSPGs)** are elevated in the glial scar and are a major deterrent to successful regeneration. To develop a more efficient method to accomplish CSPG degradation (than the current use of the bacterial enzyme chondroitinase), we are studying a normally occurring catabolic protein for CSPG degradation, the neural aggrecanase, ADAMTS-4. **Scope:** We are using a two prong approach – employing studies both *in vitro*, and *in vivo*. We are isolating, purifying and testing aggrecanase on astrocytes in tissue culture; and we are testing aggrecanase lentiviral constructs in an injury model *in vivo*, alone and in combination with chondroitinase, to ameliorate CSPG-induced inhibition. Studies conducted to date show a strong correlation between treatments with ADAMTS-4 and reduced sensory and motor behavioral deficits following a dorsal SCI in a rodent model.

The following is a Progress Report for CDMRP grant SC090248, entitled "A Better Way to Excise Inhibitory Molecules (CSPGs) from a Spinal Cord Injury Scar to Promote Regeneration". Each point of progress is addressed in relationship to the Statement of Work provided in the original application.

Statement of Work

Overarching goal: To successfully degrade aggrecan and related CSPGs using naturally occurring ADAMTS-4, alone and in combination with the current "gold standard" (chondroitinase) to attenuate axonal inhibition, and promote plasticity and regeneration of adult neurons of the CNS.

Task 1. We will determine if aggrecanase and chondroitinase-mediated degradation of CSPGs produced by primary rat cortical astrocytes will foster neurite outgrowth *in vitro*.

1a. We will confirm that primary astrocytes upregulate CSPGs *in vitro* in response to injury (experimentally induced by trauma and/or administration of TGF-beta (Smith and Strunz, 2006). We will also catalogue the specific PGs upregulated and their time course (months 1-4).

In the previous year (as presented in the 2012 Progress Report and summarized here), we did the following: Neonatal rat cortical astrocyte cultures were incubated with TGF- β (to induce injury), medium was collected and prepared for analysis of total cellular RNA, to determine quantitative real time PCR analysis of proteoglycan mRNA. Both medium and cell layer samples were chromatographed on Sephadex G-50 then applied to a column of DEAE Sephacel. Chromatography of medium and cell layers from astrocyte cultures each revealed several peaks that were pooled and concentrated. Dot blot analyses of pooled fractions using antibodies specific for different CSPGs were performed and reacted with antibodies to aggrecan, neurocan, phosphacan, NG2, brevican, versican, G3 domain of aggrecan, and aggrecan CS-2 domain aggrecanase-generated neopeptides GELE and KEEE (mixture of both antibodies). Images to support these data were provided in the previous annual report.

Continuing onward, previous and recent analyses showed the isolated pools were largely mixtures of genetically distinct proteoglycans. We detected aggrecanase generated fragments in the major peak of medium-derived PGs eluting from the DEAE column (reacting with GELE/KEEE antibodies), which was also

reactive with an antibody to the aggrecan G3 domain (also consistent with CS---substituted fragments having an intact C-terminus, this pool being highly inhibitory to neurite outgrowth.

Newly, the PG pools were further characterized for sulfation patterns using HPLC sulfated disaccharide analyses. To summarize, samples (5-10 micrograms GAG) of astrocyte cell layer lysate pools 1-4 and astrocyte conditioned medium pools 1-3 (**Figure 1**) were sent to the Glycotechnology Core Resource at the University of California - San Diego for analysis. Initially only the major peaks, medium pool 3 (M3) and cell layer pool 2 (C2) were analyzed for monosaccharides by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using 10% of each sample. Both M3 and C2 contained a high amount of GlcNAc and a small amount of GalNAc. The low GalNAc content suggests that chondroitin sulfate (CS) is a only minor component of both pools. The high GlcNAc content of both pools suggested an abundance of heparan sulfate (HS) or keratan sulfate (KS). This result was not surprising for the C2 sample, which may be enriched in cell-associated HSPGs. In addition to GlcNAc, the M3 sample contained a small amount of fucose (Fuc) and galactose (Gal), indicating the possible presence of keratan sulfate II (KSII). CS-disaccharide analysis was performed with fluorometric detection. This analysis confirmed a higher amount of CS in the M3 sample compared to the C2 sample. The M3 and C2 samples were next analyzed for heparan sulfate disaccharides on UPLC-FL using BEH-C18 column after AMAC tagging of the disaccharides. Heparan sulfate analysis confirmed the presence of HS in both M3 and C2. There were differences in the HS disaccharide spectra between M3 and C2. Notably, there was relatively less Δ UA2S-GlcNH₂6S present in the C2 sample and more of this species in the M3 sample.

In addition to the above data, a postdoc in the laboratory Dr. Justin Beller, under separate funding, is investigating the role of sulfation in these PGs related to axonal guidance and regeneration.

Other aspects of this task were reported on previously.

1b. Using a lentiviral system, we will induce primary astrocytes to degrade CSPGs via aggrecanase, chondroitinase, or both. Degradation of CSPGs will be confirmed using an anti-C-4-S antibody (2-B-6), which indentifies CSPG stubs following chondroitinase cleavage, or by antibodies to neoepitopes that are generated when aggrecanase cleaves the CSPG protein core into specific fragments. (months 5-7).

We have generated three ADAMTS-4 expression constructs, each with a specific, useful characteristic, e.g. His-

tag for identification. Constructs have been transfected into HEK293 cells (model of human astrocytes). Results of this task were reported previously.

To produce recombinant ADAMTS-4 protein for experiments *in vitro*, or for injection into rat spinal cord injury sites (for Task 2a), we transiently transfected one of the constructs (#703; having a C-terminal FLAG tag) into HEK293T cells for protein expression, which gave high levels of recombinant protein expression. The pBOB/ADAMTS-4/FLAG construct lacks a reporter gene, so SDS-Page and Western blot analyses were used to screen for Flag tagged ADAMTS-4. The co localization of ADAMTS-4 and Flag signal indicate successful transduction and expression of ADAMTS-4 (**Figure 2.**) Further, chABC transduction was successfully done and is shown by a GFP reporter gene (green fluorescence) in a confluent astrocytes monolayer (**Figure 3**).

We developed a lentiviral expression system that enables the inducible expression of ADAMTS4 in primary cultured astrocytes, as well as inducible expression *in vivo*, in a rat model of spinal cord injury, but this system was fraught with problems (some we overcame), but good practices dictated we abandon this direction. We are currently using only the His-tagged expression construct to accomplish our goals both *in vitro* and *in vivo*.

1c. Using the above system, we will determine if aggrecanase-mediated degradation of CSPGs in primary rat cortical astrocytes induces growth-inhibiting (CSPG-producing) astrocytes to become growth permissive (CSPG-degraded), and thereby foster regeneration of adult neurons (CST, RST, DC (DRG)) (months 8-11).

As progress from our previous report, we examined PG production and enzyme degradation of PGs in a co-culture model using transduced primary neurons and primary injured astrocytes. We cultured primary rat astrocytes as described previously with and without TGF-beta (activates astrocytes and induces them to upregulate proteoglycans, i.e. injured). Primary chicken DRG neurons (E9) were grown on confluent monolayers of these astrocytes, or in sparse cultures such that the neurons adhered to laminin or PLO first, then encountered transfected astrocytes (**Figure 4**). These cultures look promising qualitatively, but are currently being analyzed for quantitative measures, e.g. neurite lengths.

1d. We will test the responses of other neurons, e.g. 5HT, which have been shown to be robust following SCI in previous studies (months 12-13). Using the NS-1 neurite outgrowth assay, we will determine CSPG production (ELISA), CSPG cleavage (Western blot analyses), and aggrecanase activity (enzyme activity assays and immunostaining for neoepitopes) (Miwa, Gerken et al. 2006; Miwa, Gerken et al. 2006). Further, we will

isolate aggrecanase-generated fragments and test their effect(s) on elongating axons in vitro.

These assays, although partially automated, are highly time consuming. Thus, we are limiting our analyses to only DRG neurons, the most relevant of the neuron types listed for this study, and the one to which we have the most data for comparison.

Task 2. We will determine if aggrecanase-mediated degradation of CSPGs produced *in vivo*, in a rat spinal cord injury model system, will permit regeneration (months 13-36), and the effects of combining aggrecanase and chondroitinase treatments.

2a. Lentiviral transfection of ADAMTS-4 in an SCI model system will be performed, using currently approved methods (IACUC Protocol #2010-0702; approved 8-18-14). We will transduce, using a lentiviral vector, ADAMTS-4, within an injured region of the rat spinal cord (dorsal hemisection), (months 13-18).

Lentiviral preparation was described in the previous report (2012).

In preparation for injection *in vivo*, it was necessary to show the aggrecanase was active. We used both active and heat-inactivated aggrecanase for digestion of recombinant aggrecan, the results of which are shown in **Figure 5**. Recombinant aggrecan (40ng) was digested in the absence (lane 1) or presence (lanes 2-5) of recombinant ADAMTS-4 (15 μ l of 5mg/ml stock) at 37 °C. Incubation times ranged from 2 hours (lane 2), 12 hours (lane 3) to 24 hours (lanes 4, 5). Recombinant ADAMTS-4 was heat-inactivated by heating at 95 °C for 30 minutes (lane 5). These data show that the enzyme we were injecting *in vivo* following the dorsal hemisection spinal cord injury was active.

Surgeries. We have performed spinal cord injuries at the C-6/7 level in rat to address hand function, the focus of this study. Reproducible, consistent injuries and resulting consistent behavioral deficits are vital for proper determination of the success of our aggrecanase/chondroitinase treatments. For this reason, we have been using an injury device fabricated by colleagues at nearby University of Louisville. We demonstrated details of this procedure in the previous report (2012).

To reiterate the basic procedure here, rats received cervical SCI after learning various sensory and motor behavioral tasks. Adult male Sprague-Dawley rats (250-300g) were anesthetized with 5% isoflurane mixed with 100% oxygen at a flow rate of 4L/minute. A laminectomy at C6-C7 was performed to expose the dorsal roots.

Using the Vibraknife (SM Onifer et al., 2005; RL Hill et al., 2009), a dorsal hemisection was made to a lesion the dorsal columns (DC) and a small dorsal-most portion of the corticospinal tracts (CST). At the time of injury, a thin silicone tube with an osmotic mini-pump (Alzet model 2002, Durect Corp) was inserted into the subarachnoid cavity using a surgical microscope. The parameters for the pump were 200-250 ul solution, 0.5 ul/hr and 14-d delivery time. The solutions administered were recombinant aggrecanase or aggrecanase LV, and as a control, heat-inactivated aggrecanase. The tube was sutured to the spinous process with Vetbond to anchor it in place and the mini pump was placed under the skin on the animals back at the position of T2. After, the muscles and skin were closed in layers and the animals were cared for according to post-surgical methods approved in protocol 2010-0702. Only 2-3 animals over the whole series of experiments had any difficulties following surgery. The well-being of the animals was tended to, and the animals were removed from the study.

Still remaining to be done under the no cost extension of this award is the combination of aggrecanase with chondroitinase, using the model above. Now that all facets are optimized, this should be very straight forward. A major goal of this study is to use combinational therapy (aggrecanase + chondroitinase (cABC)) to promote regeneration *in vivo*. One setback for this study was that our aggrecanase lentivirus was prepared using a different backbone than the chondroitinase lentiviral vector, supplied by the Smith lab (George Smith, PhD: colleague and previous collaborator). Taking advantage of the lentivirus expertise within SCoBIRC (Charles Mashburn, PhD), our ADAMTS-4 insert has also been cloned into a second construct, pCSC-SP-PW, which matches the construct used to generate the cABC lentivirus. This protocol will result in fewer experimental variables when the reagents are used simultaneously in future experiments. As we did for aggrecanase, we have confirmed chase activity compared to a heat-inactivated control (**Figure 6**) and are now ready for combinational studies *in vivo* with aggrecanase. Series to begin following DOD approval of no cost extension. 2b. Aggrecan degradation in the lesion will be monitored with anti-neoepitope antibodies that will recognize aggrecan fragments. Using a variety of microscopy methods and established tract tracing techniques, neurons traversing the glial scar depleted of aggrecan by ADAMTS-4 will be quantified relative to untreated rats. (months 19-24).

Following 14 day infusion of aggrecanase, some animals were euthanized, and spinal cord tissue from test and control animals were screened via Western blot to confirm diffusion of ADAMTS-4 from the pump injection site. To identify aggrecanase distribution *in vivo*, we used Western blot analysis on spinal cord sections (**Figure, 7**). Distribution of ADAMTS-4 in spinal cord tissue was visualized using a C-terminal His tag. A single spinal cord was cut into 7 equal sections representing the epicenter (E), caudal sections (C1 – C3) and rostral sections (R1 –

R3) regions relative to the site of ADAMTS-4 injection (C6/7). The data show diffusion on aggrecanase into the spinal cord mainly at the epicenter and at least partially into both rostral and caudal directions.

To show that ADAMTS-4 is actively degrading aggrecan *in vivo*, we used 1° antibody anti-NITEGE (to aggrecan core neoepitopes after aggrecanase treatment) (**Figure 8, longitudinal sections; and Figure 9, transverse sections**), and higher magnification in **Figure 10**. Sections were cut on a freezing microtome (cryostat) at 20 μ m, mounted on glass slides, and stained. In Figure 8, injured rats were infused with active ADAMTS-4 for 14 days. Brown reaction product (DAB) is staining with NITEGE antibody counterstained with Hematoxylin (R = Rostral, C = Caudal, and E = Epicenter.) Samples were selected in each direction from the injury site approximately 4 mm long and including a 4 mm section around the injury site (Dorsal = up, Ventral = down.) The catheter placement for the mini pump as at C2 (near cervical 6/7). In Figure 9, cross sections (transverse) were taken of rat spinal cord from sham and animals treated with ADAMTS-4 for 14 days, then stained with anti-NITEGE. Brown reaction product indicates aggrecan core protein neoepitope, thus cleaved by aggrecanase (both endogenous and exogenous aggrecan is degraded). Figure 10 shows high magnification of sections from the cohort in Figure 9, to show matrix degradation around individual cells at the white matter/gray matter interface of the dorsal cord.

2c. Histological assays to identify all cell types and molecules of interest in vivo. (months 25---28)

Histological analyses were reported on previously (2012). Histological analyses are ongoing with analyses in vivo (see Figures 8 and 9).

2d. Behavioral assays. Repeat in vivo paradigm and test behavioral recovery using the Reach, Grasp and Pellet Retrieval test (motor), the Grid Walking test (motor), and the Sticker Attention test (sensory) (months 29-36).

Animals dedicated for behavioral studies were trained in the behavioral paradigms prior to injury and treatment. Then, two weeks after injury and aggrecanase treatment, the animals began a timed progression of behavioral assessments. All data were analyzed using repeated measures ANOVA (RM ANOVA), including the between groups factor and analysis of the interaction term. Following significant main effects of testing times, simple RM ANOVA procedures re performed within each group to compare changes in responses over time. All post hoc comparisons are performed using Tukey HSD post hoc t-tests based on the resulting

experimental sample sizes, number of comparisons, and independence of means. All studies were done blind for both experimenter and analyzer.

Chris Calulot (senior technician) is now testing the last cohort of the “aggrecanase alone” series, which will give a sample size of 12 for both the heat-inactivated and active ADAMTS-4 groups and 7 for the sham group. This data will be ready for complete analysis in about 2 weeks.

The results of the behavioral tests thus far are very encouraging! The dorsal hemisection SCI at C6/7 in rat causes an impairment of the fine mechanical sensory systems and moderate motor impairment in the forepaw. Sensation is tested using the Sticker Sensory Task (**Figure 11**), and is done only after the final motor test, due to behavioral acclimation. The graph to the left shows there is a significant increase in the time an injured rat first notices the sticker placed on its paw. However, treatment with a recombinant aggrecanase abolishes this increase and returns it to an interval not statistically different from uninjured animals. The graph to the left shows that over a course of 5 trials, the injured animals (both those receiving active aggrecanase and those not) are showing reduced latency though not returning to uninjured sham levels. However, a trend is appearing suggesting that aggrecanase treatment is reducing the amount of time till first notice in the initial trials, likely before the animals get acclimated to the test. **Figure 12** shows results of the Staircase Retrieval task, an indication of forepaw use for reaching, grasping and retrieving food to the endpoint of successful consumption of food pellets (see grant for details of behavioral assay.) These data show significant improvement in the Staircase Retrieval test with 14-day aggrecanase administration.

Personnel:

Previous collaborators on the study were Dr. Thomas Hering, Case Western Reserve University, Cleveland, OH, and Dr. Stephen Onifer, now at Palmer Rehabilitation Center in Davenport, IA. Both investigators remain committed to consulting for this study, but have not been active in research in the progress report period.

FIGURES

Figure 1. Chromatographic analysis of carbohydrate composition from astrocyte pools.

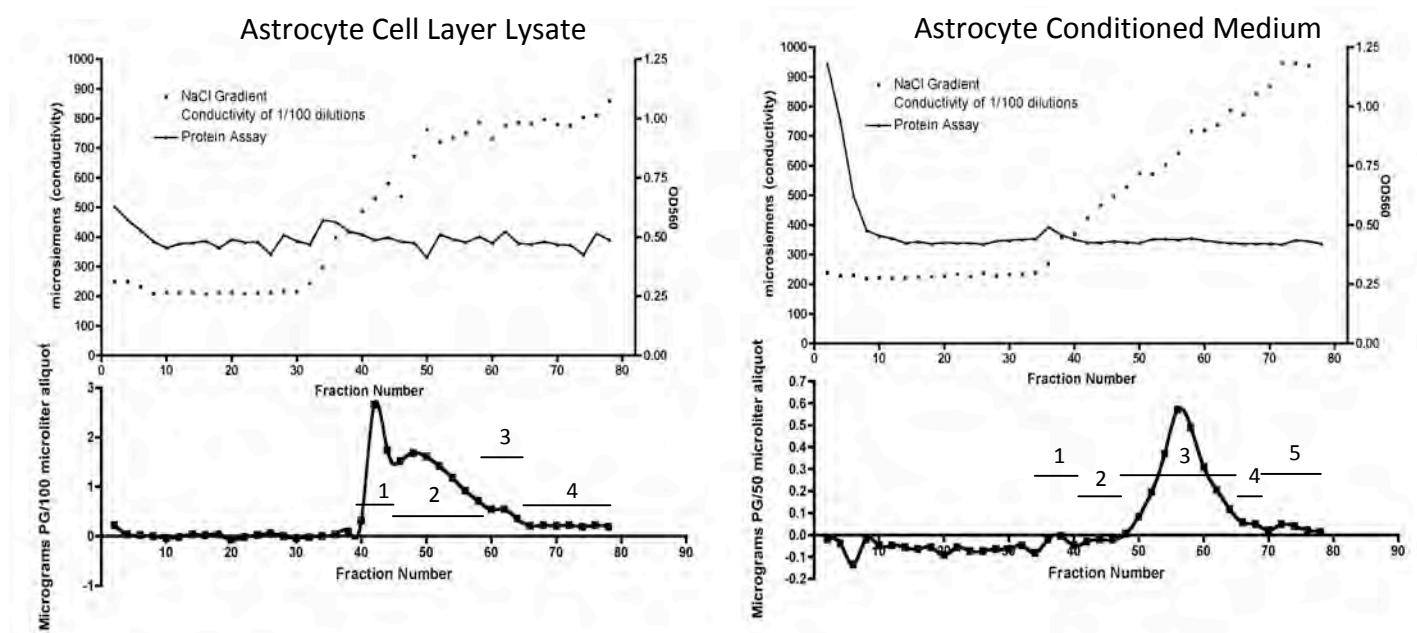


Figure 2. Media samples from astrocytes transduced with pBOB/ADAMTS-4, probed with FLAG-M2 @ 1:2000. Co-localization of ADAMTS-4 and Flag signal indicate successful transduction and expression of ADAMTS-4.

Exp 186: media samples from astrocytes transduced with pBOB/ADAMTS-4, reprobbed with FLAG-M2 @ 1:2000

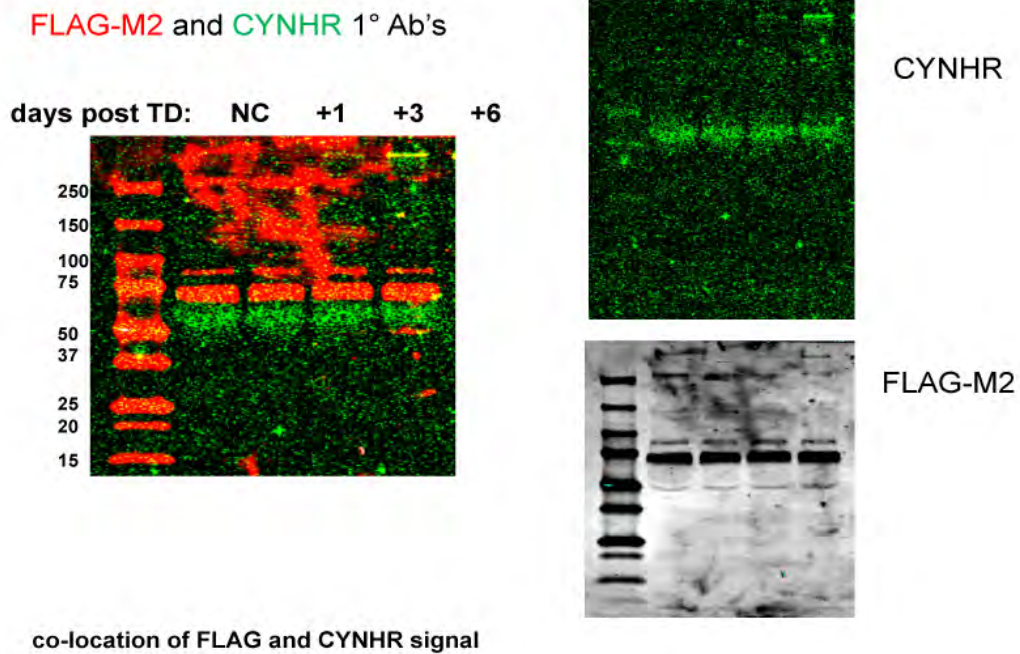


Figure 3. Rat primary astrocytes transduced with pBOB/chABC/GFP lentivirus.

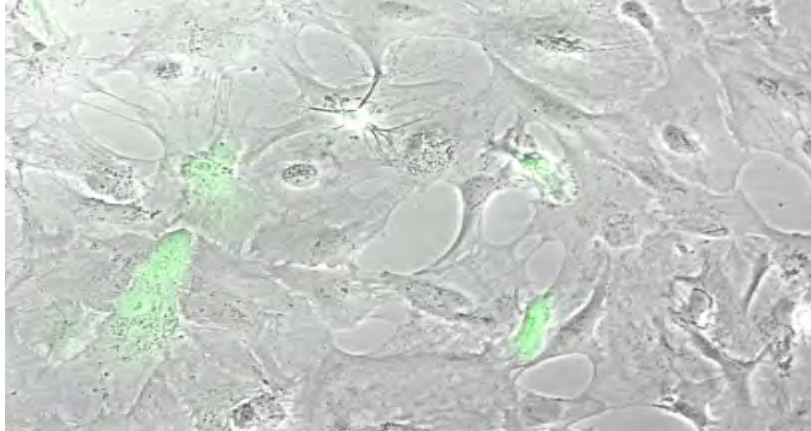


Figure 4. Representative image of DRG neurons and astrocytes in co-culture, demonstrating the basic paradigm. Rat primary astrocytes are cultured in the presence of TGF β (5ng/ml, 48 H), then transformed overnight (to over or under express a variety of CSPGs), followed by seeding of chick DRG neurons (E9). The co-cultures are then fixed and labeled with β III-tubulin (TRITC) to image neurons, and GFAP (Cy5) 1° Ab's to image astrocytes, and DAPI to label all nuclei. (40X). Quantitative analyses are ongoing.

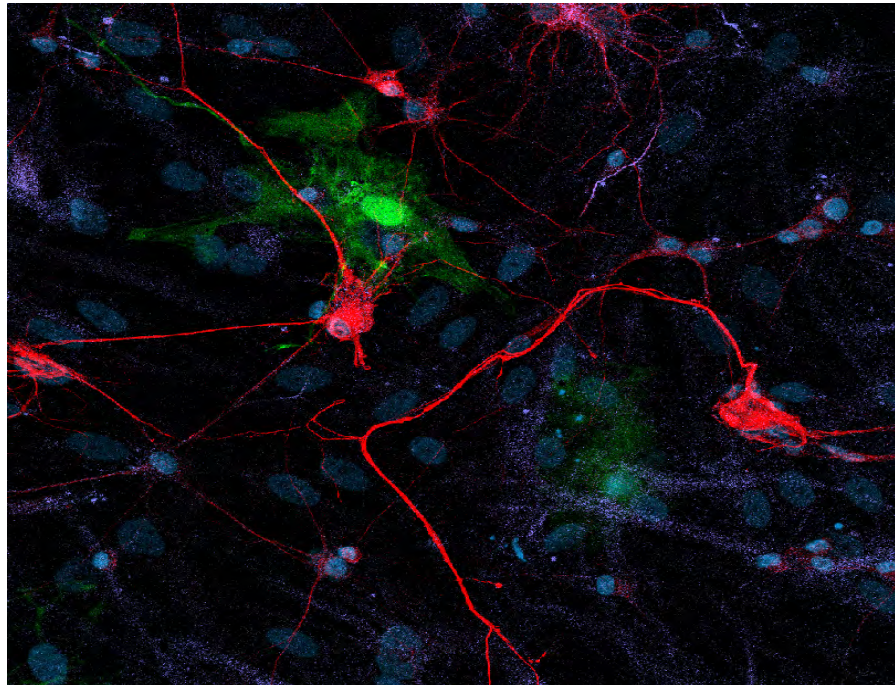


Figure 5. Active vs HI ADAMTS-4 digestion of r-aggrecan. r-Aggrecan (40ng) was digested in the absence (lane 1) or presence (lanes 2-5) of r-ADAMTS-4 (15 μ l of 5mg/ml stock) at 37 °C. Incubation times ranged from 2 hours (lane 2), 12 hours (lane 3) or 24 hours (lanes 1, 4, 5). r-ADAMTS-4 was heat-inactivated by heating at 95 °C for 30 minutes (lane 5).

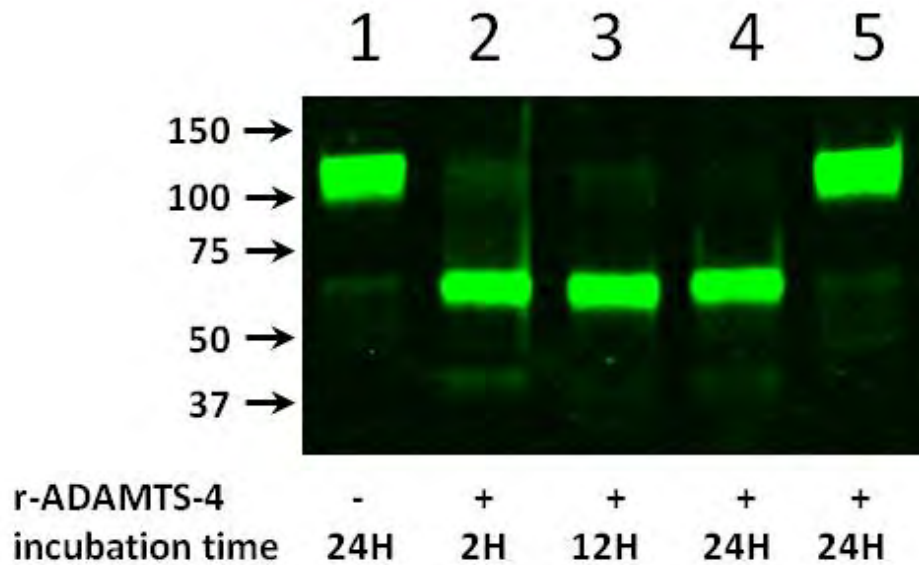


Figure 6. Chondroitinase ABC activity assay. Degredation confirmed using 1° Ab clone 3-B-3 to C-6-S stubs revealed following enzyme degradation.

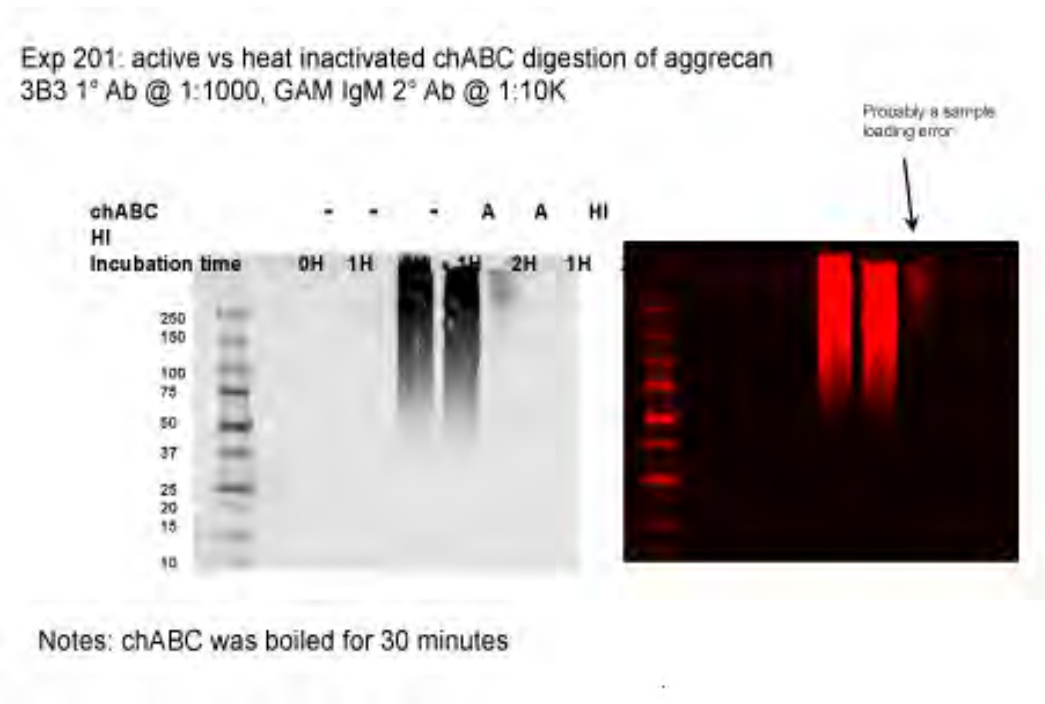


Figure 7. Distribution of recombinant ADAMTS-4 in spinal cord tissue as visualized by a C-terminal His tag. A single spinal cord section has been cut into 7 equal sections representing the epicenter (E), caudal (C1 – C3) and rostral (R1 –R3) regions relative to the site of r-ADAMTS-4 injection.

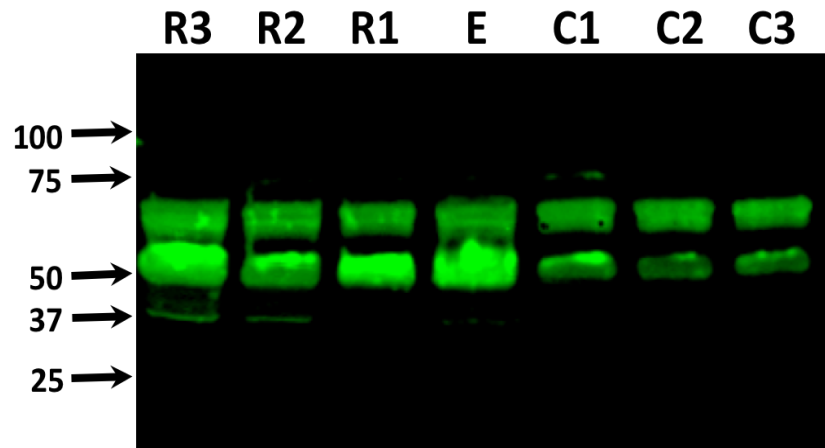


Figure 8. Injured rat infused with ACTIVE ADAMTS-4 for 14 days. Brown is staining with NITEGE antibody counterstained with Hematoxylin. R = Rostral, C = Caudal, and E = Epicenter. I took two sections in each direction from the injury site approximately 4 mm long and including a 4 mm section around the injury site. Dorsal = up, Ventral = down. The catheter placement for the mini pump is at C2 (near cervical 6/7).



Figure 9. Cross section of rat spinal cord from sham and animals treated with ADAMTS-4 for 14 days, then stained with antibody NITEGE, directed to an epitope on cleaved aggrecanase protein core. DAB reaction product (brown) indicates aggrecan core protein neoepitope, thus cleaved by aggrecanase.

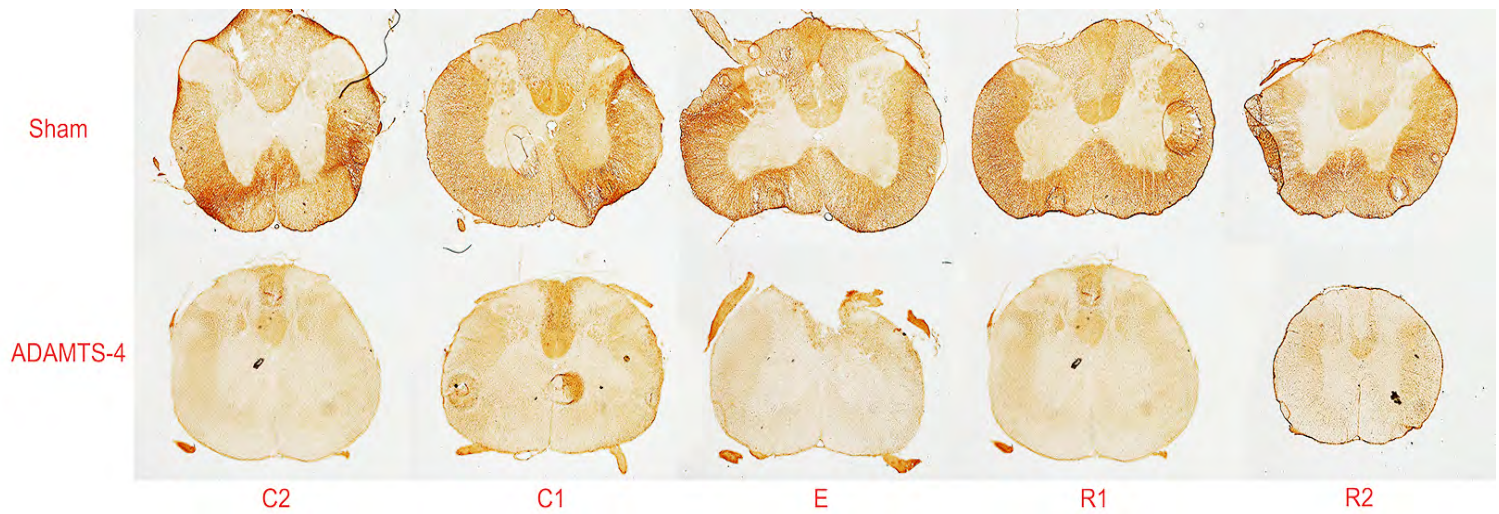


Figure 10. High magnification of images shown in Figure 9. Note the intense reaction product, i.e. degradation of aggrecan (blue arrows), surrounding individual cells at the white matter/gray matter interface of the dorsal horn of the spinal cord where DRG axons are extending in treated animals not present in sham (control). Image at right indicates the region from which the images on the left were taken; see Figures 8 and 9 for location of C1, E and R1.

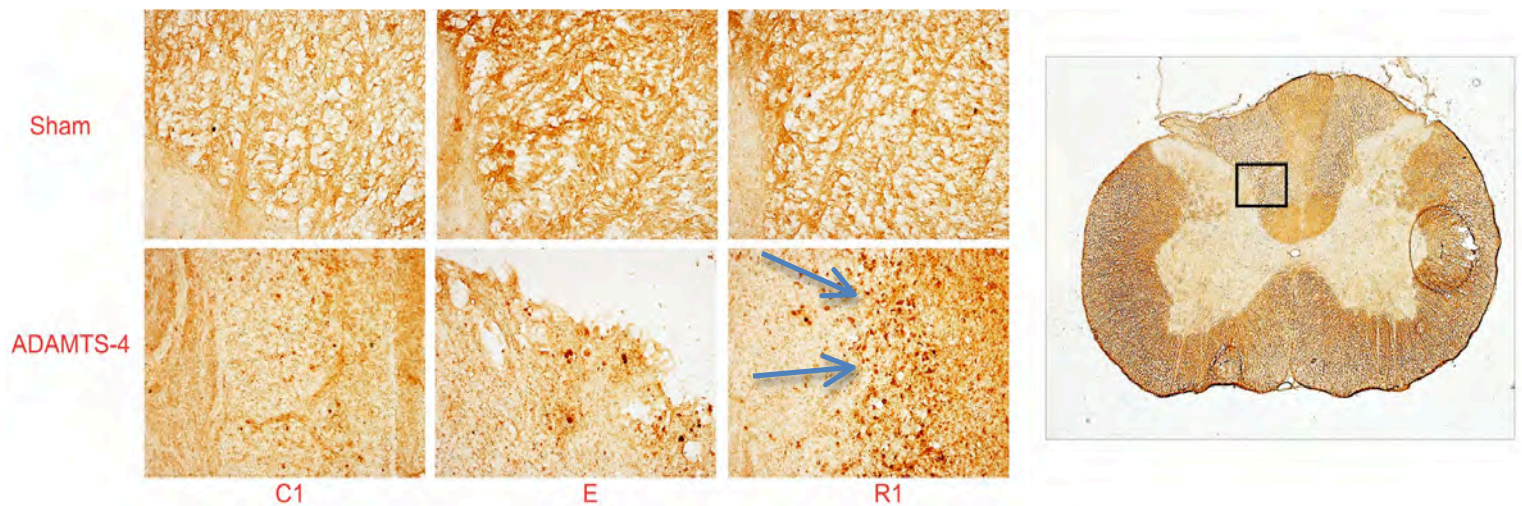


Figure 11. Significant improvement in Sticker Sensory Task results following ADAMTS-4 treatment.

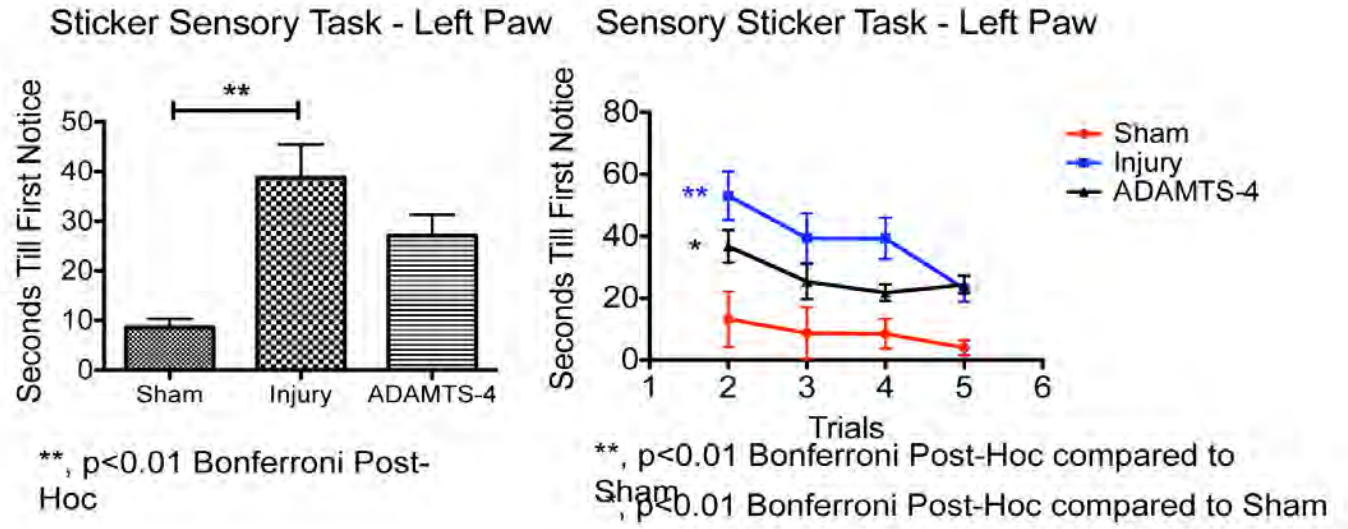
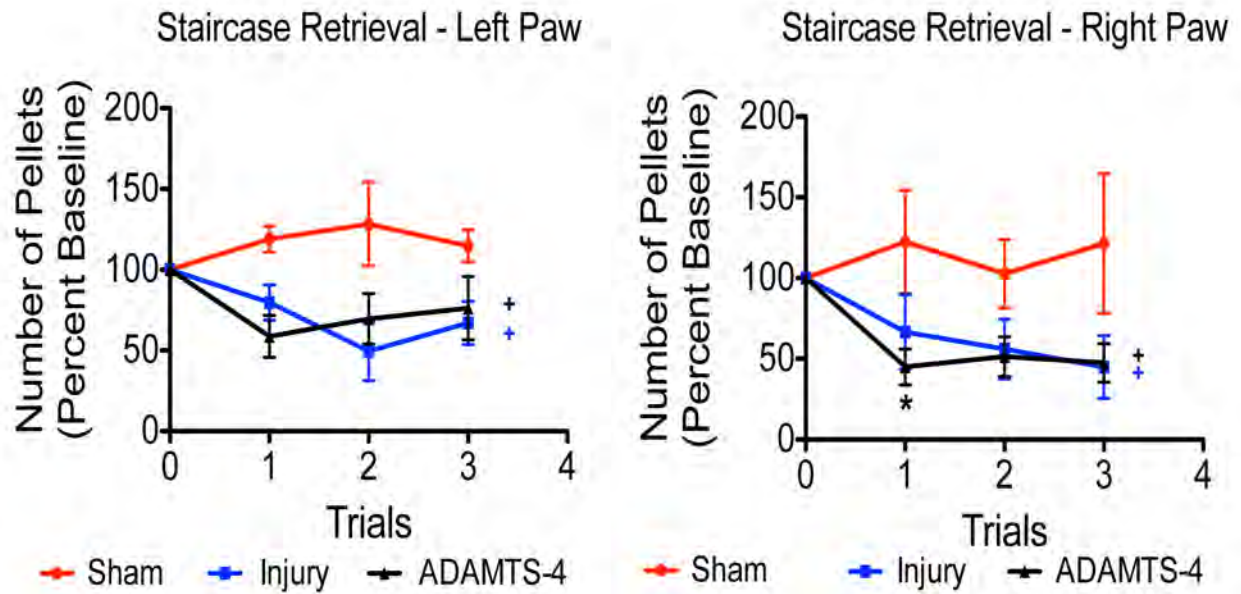


Figure 12. Significant improvement in Staircase Motor Task for reaching, grasping and retrieving food pellets following ADAMTS-4 treatment.



Key research accomplishments

Provided to us in the progress report review following submission of our year one annual report was the statement: “The PI should note that project milestones, such as completing proposed experiments or recruiting participants, are not acceptable as key research accomplishments. The Key Research Accomplishments section should be a bulleted list of important research findings resulting from the achievement of project milestones”. The majority of our achievements, by this definition, relate to the accomplishment of the statements of work.

Two previous related Key Research Accomplishments were our discoveries that:

- Removal of KS chains further enhanced NS-1 outgrowth, beyond that of chondroitinase treatment alone; and
- Further degradation with peptide-N-glycosidase F, which cleaves N-linked oligosaccharides from the core protein, resulted in a further enhancement of NS-1 neurite outgrowth

Two current Key Research Accomplishments, the second being quite major and an indicator of success, to add to this growing list are:

- Unexpected delineation of the parameters for aggrecanase diffusion into living spinal cord tissue, when administered at a Vibraknife lesion site, which will help define future experiments for any laboratory using this technology
- Aggrecanase treatment reduces the severity of injury deficits following C6/7 spinal cord injury, a major goal of this line of experimentation.

These findings are novel and exciting, and are likely to have an important impact on the field of regeneration research. They are being presented both at UK (Nov. 2013), and at the International Society for Neuroregeneration Research at Asilomar, CA in Dec. 2013.

Reportable outcomes.

Abstracts related to DOD grant since previous Progress Report:

Beller JA, Calulot CM, Hering TM, **Snow DM**, 2012. Post-translational modifications of aggrecan and inhibition of neurite outgrowth. Journal of Neurotrauma (Nat'l Neurotrauma Soc. meeting), July 2012.

Beller, JA, Hering, TM, Calulot, CM, and Snow, DM. A Novel High-Throughput Neurite Outgrowth Assay: Enzymatic Digestion Reveals Contribution of Post-Translational Modifications of Aggrecan on Neurite Inhibition. 19th Annual Kentucky Spinal Cord & Head Injury Research Trust Symposium, Louisville, Kentucky, May 6, 2013.

Beller, JA, Hering, TM, Calulot, CM, and Snow, DM. A Novel High-Throughput Neurite Outgrowth Assay: Enzymatic Digestion Reveals Contribution of Post-Translational Modifications of Aggrecan on Neurite Inhibition. National Neurotrauma Society meeting, August 2013.

Conclusions

Importance of implications of completed research: We have generated the specific tools and methodologies necessary to test aggrecanase and chondroitinase *in vitro* and *in vivo*. We have done studies to optimize the in vivo model and enzyme delivery methods. We have introduce aggrecanase in vivo using a dorsal hemisection injury model in the rodent and are currently processing tissues for observation of cellular and molecular changes, while also assessing short and longer term behavioral results.

Recommended changes: No recommended changes beyond those already implemented (see Body).

“So what?” (evaluate knowledge gained as a scientific or medical product): No scientific or medical product is evident, although the benefits of using aggrecanase treatment to reduce deficits following dorsal SCI are evident, which is a sizeable “so what” factor.

a. References

References from original application; still applicable. Not all references listed here are cited in progress report, but all in progress report are listed here.

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23. Tom VJ, Sandrow-Feinberg HR, Miller K, Santi L, Connors T, Lemay MA, Houle JD: **Combining peripheral nerve grafts and chondroitinase promotes functional axonal regeneration in the chronically injured spinal cord.** *J Neurosci* 2009, **29**(47):14881-14890.
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Appendices.

Snow, D. M., Updated Biosketch

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.

Follow this format for each person. DO NOT EXCEED FOUR PAGES

NAME Diane M. Snow, PhD	POSITION TITLE Professor with Tenure		
eRA COMMONS USER NAME <u>Diane.Snow</u>			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
The University of Akron, Akron, OH	B.S.	1982	Biology/German
The University of Akron and NEOUCOM	M.S.	1985	Neuroscience
Case Western Reserve University, Cleveland,	Ph.D.	1989	Neuroscience
Case Western Reserve University, Cleveland,	PostDoc	1990	Neuroscience

A. Personal Statement

This CV serves as an update for a Progress Report to the DOD;

“A Better Way to Excise Inhibitory Molecules (CSPGs) from a Spinal Cord Injury Scar to Promote Regeneration” SC090248

B. Positions and Honors

1984-1986	Technician, Cleveland Clinic Foundation, Dept. of Brain and Vascular Research
1986-1990	Brumagin Fellowship for Spinal Cord Injury Research
1991-1994	NIH National Research Service Award, F32
1992-1993	Macalester College, Minneapolis, MN, Faculty
1994-1996	Research Assistant Professor, The University of Minnesota, Dept. of Cell Biology and Neuroanatomy, Minneapolis, MN
1996-2002	Assistant Professor , Dept. of Anatomy and Neurobiology, The Univ. of Kentucky, Lexington, KY
2002-2008	Associate Professor with Tenure , Dept. of Anatomy and Neurobiology, The Univ. of Kentucky, Lexington
2003-04	Wethington Research Award
2001-2007	Faculty Associate, Spinal Cord and Brain Injury Research Center , Univ. of KY, Lexington, KY
2007	National ACE Network Leadership Award (Advancement of Women in Higher Education) for “an outstanding, innovative, and visionary leadership program – <i>Circles of Power</i> ”
2008	Kentucky Academy of Sciences - <i>Superlative Award, Distinguished University Scientist</i>
2008-pres	Professor and Endowed Chair , Spinal Cord and Brain Injury Research Center (SCoBIRC); Dept of Anat and Neurobiology, The University of Kentucky, Lexington, KY
2007-11	Wethington Research Award
2008; 09	Abraham Flexner Master Educator Award <i>(2011 pending)</i>

- 2009 Society for the Promotion of Undergraduate Research (SPUR) - *Excellent Undergraduate Research Mentor Award*
- 2010 Sarah Bennett Holmes Award, contribution to development of women professionals, *Women's Forum*
- 2011-12 Southeast Conference Academic Consortium – Administrative Leadership Development Program Fellow
- 2011-pres Board of Directors and Advisory Board member, Exomedicine Institute, NASA-funded zero-gravity medical research
- 2012-pres Advisory Board, *Living Arts and Science Center*, Lexington, KY
- 2013-pres Member, Omicron Delta Kappa leadership society

C. Peer-Reviewed Publications (since 2002)

- 24. **Snow, D. M.**, Smith, J.D., and. Gurwell, J. A. (2002), Binding characteristics of chondroitin sulfate proteoglycans and laminin-1, and correlative neurite outgrowth behaviors in a standard tissue culture choice assay. *J. Neurobiol* 51:285-301.
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- 28. **Snow, D.M.**, Smith, J.D., Cunningham, A. T., McFarlin, J., and Goshorn E.C. Neurite elongation on chondroitin sulfate proteoglycans is characterized by axonal fasciculation. (2003) *Exp. Neurol.* 182:310-321.
- 29. Hynds, D.L., Spencer, M., Andres, D., and **Snow, D.M.** (2003) Rit promotes MEK-independent neurite branching in human neuroblastoma cells. – *J. Cell Sci.* 116:1925-1935.
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- 33. **Snow, D.M.**, Smith, J.D., Booze, R.M., Welch, M., and Mactutus, C.F. (2004) Cocaine-induced inhibition of outgrowth in locus coeruleus neurons is related to gestational exposure period and offspring sex. *Int'l J. Develop. Neurosci.* 22:297-308.
- 34. Dey, S., Booze, R.M., Mactutus, C.F. and **Snow, D.M.** (2006) Specificity of prenatal cocaine on inhibition of locus coeruleus neurite outgrowth. *Neurosci.* 139(3):899-907.

35. Booze, R.M., Wallace, D.R., Silvers, J.M., Strupp, B.J., **Snow, D.M.**, and Mactutus, C.F. (2006) Prenatal cocaine exposure alters alpha2 receptor expression in adolescent rats. *BMC Neuroscience* 2006, 7:33
36. Heron, P., Sutton, B., Curinga, G.M., Smith, G. M. and **Snow, D.M.** (2007) Localized gene expression of guidance molecules in a co-culture model to direct axonal growth. *J. Neurosci. Meth.*, 159(2):203-214.
37. Dey, S., Mactutus, C.F., Booze, R.M., and Snow, D.M. (2007) Prenatal cocaine exposure induces apoptosis in locus coeruleus neurons *in vitro* by altering the bax/bcl-2 ratio and through caspase-3 apoptotic signaling. *Neurosci.* 144(2):509-521.
38. Curinga, G.M., **Snow, D.M.**, Mashburn, C., Kohler, K., Thobaben, R., Caggiano, A.O., Smith, G.M. (2007) Mammalian-produced chondroitinase AC mitigates axon inhibition by chondroitin sulfate proteoglycans. *J Neurochem.* 102(1):275-288.39.
39. Dey, S. and **Snow, D. M.** (2007) Prenatal cocaine exposure induces apoptosis in locus coeruleus neurons through TNF-a mediated induction of Bax and JNK-1-MAPK pathways. *J Neurochem.* 103(2):542-556.
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43. **Snow, DM** (2011) "Pioneering studies on the mechanisms of axonal regeneration. *Dev Neurobiol.* 71(9):785-9. PMID: 21938765.
44. Lin S, Liu M, Son YJ, Timothy Himes B, **Snow DM**, Yu W, Baas PW. (2011). Inhibition of Kinesin-5, a microtubule-based motor protein, as a strategy for enhancing regeneration of adult axons. *Traffic* 12(3):269-86. doi: 10.1111/j.1600-0854.2010.01152.x. Epub 2011 Jan 14.
45. Beller JA, Hering TM, **Snow DM**. "Techniques For Examining The Effect Of Substrate Bound Proteoglycans On Neurite Outgrowth In Vitro." *Neuromethods: Extracellular Matrix*. Humana Press (In Press)
46. Beller JA, Hering TM, **Snow DM**. "High-throughput Quantitative Assay for Analyzing Neurite Outgrowth on a Uniform Substratum: The Cell-Substratum Assay." *Neuromethods: Extracellular Matrix*. Humana Press (In Press)
47. Beller, J.A., Kulengowski, B., Kobraei, E., Curinga, G.M., Calulot, CM, Bahrami, A., Hering, TM and **Snow, DM**. Comparison of sensory neuron growth cone and filipodial responses to structurally

diverse aggrecan variants, *in vitro*. *Exp. Neurol.* (2013)
<http://dx.doi.org/10.1016/j.expneurol.2013.02.012>.

48. Beller, JA, Hering, TM, Calulot, C, Centers, AP, **Snow, DM.** (2013). A novel high-throughput method for measuring neurite outgrowth to exposure of substrate bound molecules: Enzymatic digestion of aggrecan yields novel role of N-glycosylation. *Invited –Neural Regeneration Research; Special Issue; Ed. D. Snow – “Neuronal Growth Cone Guidance and Extracellular Matrix Molecules”*.

D. Research Support (since 2008)

“Recombinant aggrecan variants having specifically modified CS chains and their regulation of axonal regeneration” - ACTIVE

P.I. D. Snow, 1/15/11-1/14/13

Agency: Kentucky Spinal Cord and Head Injury Research Trust

Aims: 1) To use shRNA to knock down specific biosynthetic enzymes in the CS synthesis pathway to produce modified CS chains on recombinant aggrecan (a CSPG). These specifically modified CS chains will be analyzed to confirm the nature of the predicted modification; and, 2) To determine the ability of CSPGs engineered in Aim 1 to inhibit neurite outgrowth.

"A Better Way to Excise Inhibitory Molecules (CSPGs) from a Spinal Cord Injury Scar to Promote Regeneration" - ACTIVE

P.I. D. Snow, 9/30/10-9/29/13

Agency: Dept of Defense – DOD-CDMRP

Aims: 1) To determine if aggrecanase and chondroitinase-mediated degradation of CSPGs, either purified, or produced by primary rat cortical astrocytes, will foster neurite outgrowth in vitro; and 2) To determine if aggrecanase and chondroitinase-mediated degradation of CSPGs in a rat spinal cord injury model in vivo, will result in improved axonal regeneration and recovery of function.

“Designer PGs for CNS Injury” - ACTIVE

P.I. D. Snow, 9/1/07-8/31/13 (NCE)

Agency: NIH, NINDS; 1R01NS053470-01A2

Aims: Identification and manipulation of inhibitory microdomains of glial scar chondroitin sulfate proteoglycans in vitro focusing on cortical astrocytes interactions with sensory neurons.

"Role of TRPV1 in Airway Hypersensitivity Induced by Inflammation"

PI: Lu-Yuan Lee; Co-I: D. Snow, 9/1/09 - 8/31/11

Agency: NIH - National Heart, Lung and Blood Institute; 1R01 (HL096914-01)

Aims: To investigate the role of TRPV1 in the development of airway hypersensitivity when chronic airway inflammation is induced by allergen sensitization.

“Designer PGs for Spinal Cord Injury”

PI - D. Snow; 11-1-07 to 10-31-010

Agency: Christopher and Dana Reeve Foundation

Aims: identification and manipulation of inhibitory microdomains of glial scar neurocan in vitro and in vivo using shRNA technology.

“PGs, Monastrol, and Regeneration”

P.I.s - D. M. Snow and P. Baas, 12-31-08 to 12-30-09

Agency: Christopher and Dana Reeve Foundation

Aims: To determine if the kinesin-5 inhibitor, monastrol, attenuates CSPG-induced axonal inhibition.